IN VITRO RESPONSE OF *PHOMOPSIS THEAE* TO THE PRODUCTS OF *AZADIRACHTA INDICA* AND EXTRACTS OF *WARBURGIA UGANDENSIS*

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DECLARATION

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DEDICATION

This research work is dedicated to my daughter Abigail, and my loving parents for the endless support they gave me throughout my study period.

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ABBREVIATIONS AND ACRONYMS

ANOVA	Analysis of Variance
COC	Copper Oxychloride
EC	Emulsifying Concentrate
FAO	Food and Agricultural Organization
GDP	Gross Domestic Product
ITC	International Tea Committee
KTGA	Kenya Tea Growers Association
MEA	Malt Extract Agar
NRC	National Research Council
PDA	Potato Dextrose Agar
SGM	The Society for General Microbiology
TBK	Tea Board of Kenya
TRFK	Tea Research Foundation of Kenya

ABSTRACT

Tea is one of the major cash crops in Kenya and a means of livelihood to people living around the region where it is grown. Branch and collar canker disease is among the many diseases which affect the crop hence reducing its production. Methods used to control the disease mainly involve the use of chemical fungicides which are toxic and not environmentally friendly. This study was therefore done to determine the use of plant products and extracts to manage the disease. Among the products used were Nimbecidine and Trilogy which are products of neem tree. The *Warburgia ugandensis* extracts from the bark, root and leaf were also used *in vitro* to determine the inhibition of *Phomopsis theae* the causative agent of Branch and Collar Canker. These were compared with the inhibition of standard fungicides (Topsin and Saaf). Nimbecidine and Trilogy were also tested at the concentration of 10 ppm, 25 ppm, 50 ppm and 100 ppm, while *W. ugandensis* extracts were tested at the rates of 10 g/100 ml, 15 g/100 ml and 20 g/100 ml. Nimbecidine inhibited growth more than Trilogy in all the concentrations and were not significantly different from those of Topsin and Saaf. Stem bark extracts of *W. ugandensis* were also effective in inhibiting the growth of *P. theae* with inhibition of 97.64 per cent in all the rates. Root was next in inhibition with 78.8, 19.45 and 9.89 per cent in 20 g, 15 g and 10 g respectively. The leaf extracts did not inhibit growth at any rate. In liquid media, similar results were observed. In Nimbecidine, mycelial weights were significantly lower compared to Trilogy. Stem bark extracts also had lower mycelial weights, followed by the root and then leaf among the extracts. Nimbecidine and bark extracts of *W. ugandensis* were compared with standard fungicides, Topsin and Saaf, and the extract was comparable to the fungicides both in solid and liquid media. They were able to inhibit the growth of *P. theae.*

CHAPTER ONE

INTRODUCTION

1.1 Background

Tea (*Camellia sinensis* Kuntze (L.) O history in Kenya can be traced back to 1903 when G. W. L. Caine, a European settler introduced the first seedlings from India and planted them in Limuru near Nairobi (TBK, 2010). Tea production is by smallholders and in large estates such as Brooke Bond, African Highlands and Eastern Produce Limited. The large plantations are organized under the Kenya Tea Growers Association (K.T.G.A) and account for about 40% of Kenya Tea Production. Tea is a very important revenue earner for the tea producing countries in the world. For instance, tea annually contributes about 26 percent to Kenya's export earnings and four percent of the Gross Domestic Product (GDP) (Wachira and Ronno, 2004). In 2007, Kenya exported over 360 million kilograms of processed tea which earned the country over Kshs. 43 billion in form of foreign exchange (TBK, 2008). Since the introduction of tea in Kenya, it has steadily continued to expand in acreage under cultivation with the current land occupied by the crop reaching 149,196 hectares by 2007 (TBK, 2007). The sector offers employment all-year round to about 639,521 growers in the rural areas in addition to providing employment in other tea handling processes. As a labour intensive industry, tea sector supports livelihoods of more than three million persons directly and indirectly (TBK, 2008).

Tea growing regions in Kenya are found in the Great Rift Valley. In the East of Rift are the cool Arberdare highlands and the panoramic Nyabene hills while in the West of Rift defined by the Mau escarpment are Nandi hills, highlands around Kericho, Mt. Elgon and the Kisii highlands. It is on the slopes of these highlands within the altitudes of 1500 m to

2700 m above sea level where tea is grown. These regions are endowed with an ideal climate for tea growing. The tropical, volcanic soils rich in nutrients give tea a unique flavor and character. The rainfall ranges between 1200 mm and 2700 mm annually (TRFK, 2002).

Despite its importance, tea production is facing some challenges. Sane, 2008 reported that tea production costs (labour, fuel and electricity), mismanagement, age of tea bushes, high overhead costs, bad agricultural practices, climate change and dilapidated roads that hampers quick and efficient transportation of tea to the market. Fragmentation of tea farms in the face of tumbling global prices and rising production costs is threaten the survival of Kenya's small scale tea growers (Mburu, 2008). Apart from all the affore-outlined challenges, there is also the problem of diseases. The major diseases in Africa are Amillaria root rot (*Amillaria heiminii*) and wood rot (*Hypoxylon serpens*) (Onsando *et al*., 1997). Others that include Branch and Collar Canker (*Phomopsis theae* (*P. theae*), Brown Blight (*Colletotrichum coccodes*) and Gray Blight (*Pestaliopsis theae*) are of variable importance (Anon, 1991).

The losses in terms of quantity caused by branch and collar canker have not been quantified and they are substantial (Otieno, 1998). The pathogen affects carbohydrate metabolism resulting in stunting rosettting and enhancement of purpling due to excessive accumulation of anthocyanins. Despite its economic impact, effective preventive measures are unavailable other than pruning of healthy wood and application of copper fungicides on prune cuts (Ponmurugan *et al*., 2006). Random and extreme use of

chemical fungicides for seed and soil management has led to the increase of pathogen resistance (Daghman *et al*., 2006). For instance, benzimidazole fungicides were very effective in controlling Gray Blight of tea but isolates resistant to the fungicides have emerged in most cultivation areas in Kenya.

Trilogy (clarified neem oil) is known to have antifungal activity (Moline and Lock, 1993). According to Mirza *et al*., 2000, neem products were found highly effective at different stages of *Phytopthora infestants*. Kazmi *et al*., 1995, reported that 0.1 % neem oil causes significant reduction on growth of *Alternaria alternata* and *Aspergillus* sp, Locke 1995 reported that in the field, *Alternaria alternata, Aspergillus niger* and *Fusarium oxysporum* have been controlled completely using 2-10 % neem oil. According to Niaz and Kazmi (2005), neem oil was quite effective against *Aspergillus* spp. There was a need to check if neem oil is also effective against *Phomopsis theae*, such a study has not been done before.

1.2 Statement of the Problem

Branch and Collar canker caused by *P. theae* is a disease that infects tea in newly planted fields as well as in replanted fields leading to capital losses to farmers (Ponmurugan *et al.*, 2006) Copper-based fungicides are principally used to control the disease, but they are expensive, detrimental to health and contaminate the environment. Records indicate that only 0.1% of the chemicals reach the target pathogen and more than 99% contaminate the ecosystem (Sateesh, 1998). In addition synthetic agrochemical usage has resulted in the development of resistant pests and pathogens.

1.3 Justification of the Study

Farmers in tea growing areas use chemicals to manage the various diseases which affect their tea. Such chemicals have financial and environmental implications. It is therefore necessary to search for cost-effective, non-toxic, biodegradable and ecofriendly management strategy such as the use of botanicals in place of hazardous and recalcitrant synthetic chemicals (Sateesh, 1998). Studies by Oniango (2003) revealed that extracts from *Warburgia ugandensis* leaves are active against the Branch and Collar Canker pathogen. However, it is not known if extracts from other parts of *W. ugandensis* like bark and root are also active. Besides, it is also necessary to evaluate products from other plants, particularly neem for efficacy against the pathogen. Neem products and extracts have the potential as safe alternatives for chemical fungicides in plant disease management and apart from conventional fungicides and microbial agents, these have been found effective against a wide range of plant pathogens for example *Colleotrichum lindemithianum* and *Fusarium oxysporium* (Amadioha, 2003; Bowers and Locke, 2004).

1.4 Research Questions

i) Do the neem products (Trilogy and Nimbecidine) significantly suppress the growth of *P. theae*?

ii) Do the extracts from the root, bark and leaves of *W. ugandensis* significantly suppress growth of *P. theae*?

iii) Are there any effects of *W. ugandensis* and neem products on growth of *P. theae* comparable to the effects of the standard fungicides Topsin and Saaf?

1.5 Hypotheses

i) Neem products (Trilogy and Nimbecidine) do not significantly suppress the growth of *P. theae.*

ii) Bark, root and leaf extracts of *W. ugandensis* do not significantly suppress the growth of *P. theae*.

iii) The effects of neem products, *W. ugandensis* extracts and a standard fungicide Topsin on growth of *P. theae* do not differ significantly.

1.6 Objectives

1.6.1 General Objective

To determine the efficacy of *W. ugandensis* extracts and neem products against of *P. theae.*

1.6.2 Specific Objectives

i) To evaluate the effects of neem products (Trilogy and Nimbecidine) on growth of *P. theae*.

ii) To evaluate the effects of the bark, root and leaf extracts of *W. ugandensis* on the growth of *P. theae*.

iii) To compare the effects of the neem products and *W. ugandensis* extracts on growth of *P. theae* with standard fungicide Topsin and Saaf.

1.7 Significance of the Study

Results from the project provided information on the role of the products from plants such as neem and *Warburgia ugandensis* on the management of *P. theae*. It provided a

sample of data on how best some local plants can be used in integrated disease management programmes in agricultural systems. Products from these plants are environmentally friendly and easy to use. The effects of the *W. ugandensis* bark extracts on the growth of *P. theae* were like that of the standard fungicides and subsequently supplement or even replace the use of fungicides.

CHAPTER TWO

LITERATURE REVIEW

2.1 Tea

2.1.1 Description

Tea (*Camellia sinensis* (L.) O. Kuntz is a perennial plant of the Theaceae family (Cabrera *et al*., 2003). It was first cultivated in South Asia but nowadays, it is widely grown throughout Asia, Africa and various parts of the Middle East (Chopade *et al*., 2008). The two main cultivated varieties of tea are *Camellia sinensis* var. *assamica* which has relatively large leaves and *C. sinensis* var. *sinensis* with small semi-erect leaves. The tea variety, *Assamica*, originated from the forest of Assamica in northeastern India while *sinensis* originated from Sichuan province south-western China (Van der Vossen and Wessel, 2002). *Assamica* sp. prefers a semi tropical climate and on average, has a higher caffeine and catechin content than *sinensis.* Green tea is made mainly from *sinensis* and black tea from *assamica* (Astil *et al*., 2001, Singh and Ahija, 2006).

Tea plant is an evergreen shrub with large number of branches. The leaves appear glossy dark, elongate ovate, roughly serrate, alternate and short-petiolate (Greunwald, 2007). Flowers are white fragrant, 2.5- 4.0 cm in diameter and are in solitary or in clusters of two or four. They bear numerous stamens with yellow anther and produce brownish red capsules (Ross, 2005). The fruit is a flattened, smooth, rounded trigonous three celled capsule, the seeds are solitary, in each in a size of a small nut (Biswas, 2006).

Tea is the second most widely consumed beverage in the world, second only to water (Ferruzzi and Green, 2006). It is believed that the polyphenolic compounds, flavonoids,

of tea are responsible for its useful pharmacological properties (Lu *et al*., 2003). The beverage include black (aerated), green (non-aerated) and oolong (semi-aerated) teas which constitute about 78 %, 20 % and 2 % respectively of the total world production (Basu and Lucas, 2007).

2.2 Cultivation

Camellia sinensis is mainly cultivated in tropical and sub-tropical climates, in regions with fair temperature, acidic soils and highly humid environmental conditions (Dufresne and Farnworth, 2001). However, it is commercially cultivated from the equator to as far north as Cornwall in the UK mainland. Under normal conditions the tea plant is an evergreen tree that widely grows into a medium size tree, but under cultivation it is pruned and trained to a low spreading bush thus ensuring that a maximum crop of young shoots can be obtained (Hajra, 2001).

2.2.1 Climate

The plant thrives best under high and evenly distributed rainfall but within the tropics it can tolerate a dry season of not more than three months. At an altitude of 1800-2000 m, with an average annual rainfall of 1800 mm and a dry season of three months with approximately 50 mm of water, an average annual yield of 2 tons per hectare of commercial tea can be expected (TRFK, 2001). Tea requires an average of five hours of sunshine per day. In cloudy conditions and with heavy and continuous rainfall the yield drops, as it does when the weather is hot, dry and sunny. Atmospheric humidity must be high 70- 90 % (Anon, 1991). Kenya's tea growing regions straddles the equator with the economic production taking place at altitudes ranging from 1600 – 2600 m in the

highlands west of the Great Rift Valley and Mount Kenya regions. They are endowed with ideal climate, tropical, volcanic red soils, well distributed rainfall ranging between 1200 mm to 1400 mm per annum and long sunny days (TBK, 2010). Tea is cultivated in tropical and sub-tropical climates but commercial cultivation can also be found in temperate areas (den Braber *et al*., 2011). In order to increase the productivity of the enterprise, tea is grown in diverse regions of varying climates (Owour *et al.,* 2010). The ideal temperature for tea growth is 18-30 ^{o} C. Growth is limited by temperature above 32 to 35 ^{o} C and below 12-13 ^{o} C (den Braber *et al.*, 2011). Minimum and maximum temperatures have a negative correlation with the changes of total tannin content of bush tea grown in locations with different altitudes (Nchabeleng *et al*., 2012).

2.2.2 Soil Requirements

Generally, the best plantations of tea are found on deep soils with a good structure, well drained with well developed humus bearing layer and high mineral reserves. It grows in a variety of soil type such as alluvial soils, drained peat, sedimentary from gneiss and granite and soils derived from volcanic ash (Meliva and Baker, 2007). Tea prefers acidic soils with pH between 4.5 and 5.0 (Zee *et al*., 2003). Acid loving plants grown in soils with higher pH ranges can suffer from nutrient (zinc and iron) deficiencies that result in poor growth. It thrives well on well drained, permeable, deep and fertile soils with a minimum of 2 m depth (Lemmessa, 1996).

Soil temperature is also related to growth, with an optimum range, over which there is a linear relationship of 19 – 22 ^{o} C. A good depth of soil ensures that water which has

drained to a lower level can remain within reach of tea. Tea roots have been noted growing to over 15 m depth in suitable soils (Wilson, 1999). Lack of soil and atmospheric moisture decreases the growth of lower branches and leaves become hard and tend to consequently yield and quality decrease.

2.3 Tea Production

Tea is produced in over 20 countries with major producers being India, China, Sri Lanka, Kenya, Indonesia and Turkey (ITC, 2008). World production now stands at 3.2 million tones up from 2.1 million tones in 1993 while plantation coverage has risen to 140 million hectares from 105 for the same period (ITC, 2008; FAO, 2006). Since introduction of tea in Kenya, it has steadily continued to expand in acreage under cultivation with the current land occupied by the crop reaching 149,196 hectares by 2007 (TBK, 2007). In 2011, the industry export earnings amounted to Kshs. 109 billion which was higher than the total earnings of Kshs. 97 billion recorded in 2010 and Kshs. 69 billion in 2009 (TBK, 2012). There has been a marked improvement in Kenyan tea production over the years with notable replacement of the pioneer seedling tea with improved clonal cultivars that are better yielding and of good quality, and better attributes like; tolerance to drought, pests and diseases (Wachira, 2002).

The tea industry is divided into large estates and small holder sub-sectors (TRFK, 2002). The large estates are under the control of big multinational companies and account for about 40 % of total processed tea (TRFK, 2001). The small holder growers, with average holding ranging from less than one hectare to 20 hectares, are managed by Kenya Tea

Development Authority (KTDA) through individual tea processing factories (Mwaura and Muru, 2007).

2.4 Tea and Health Benefits

There is a growing body of knowledge about health benefits of tea. The knowledge has however, been largely generated from studies using non-fermented green tea (Carmen *et al.*, 2006). The many health benefits that have been ascribed to consumption of tea beverage include; reduction of cholesterol, protection against cardiovascular diseases and cancer (Zuo *et al.,* 2002). Beneficial effects of tea have been attributed to the strong oxidative activity of the tea phenolic compounds known as catechins (Zuo *et al*., 2002, Rao *et al*., 2007). Polyphenols from green as well as black tea have been associated with the amelioration of inflammation, inhibition of diabetes including hyperglcaemia (Vinson *et al*., 2004), prevention of intestinal damage and anti-diarrhoea properties, enhancement of oral health and potential to improve spatial cognitive learning ability (Haque *et al.*, 2006).

2.5 Diseases of Tea

Several diseases attack Kenyan tea (Owour *et al*., 2005). They include root rot caused by *Armillaria mellea* and Hypoxylon wood rot caused by *Hypoxylon serpens*. In Armillaria root rot, the symptoms include, bush reduction in growth, yellowing, premature flowering, defoliation and eventual death (TRFK, 2002). Copper fungicides like copper oxychloride (COC) were found to control the disease more effectively. It is also managed by use of biocontrol agent *Trichorderma* sp. Avoiding planting tea in drier areas and

planting seedlings with a well developed root system is recommended. Hypoxylon wood rot is characterized by rotten wood bearing superficial irregular dark-grey to black raised patches of fruitifications varying in size (Anon, 1991). Decline of the bush due to sectorial rooting and death of the whole plant is another consequence. The disease is managed by surgical pruning of the infected branches and the treatment with copper oxychloride mixed vegetable oil at 1:1 ratio. Blister Blight is caused by *Exosobasidium vexans*. The disease attacks young succulent and harvestable tender shoots of the plant thereby seriously affecting the quality and quantity of the harvested leaves. In the absence of any control measures direct loss due to Blister Blight could be as high as 35 % (Radhakrishan and Baby, 2004). Prekumar and Baby (2005) recommended that Blight can be managed by use of chemicals such as carbendazim, hexaconazole, propiconazole and tridemorph. Branch and Collar Canker is caused by *P. theae* which primarily affects susceptible clonal plants. The disease appears initially as a small dark brown lesion on the bark. As the lesion develops and enlarges in size the bark and cambium are killed and the necrotic area becomes slightly depressed. Damping off is caused by a fungus *Pythium* sp., which is soil borne. The fungus attacks the main stem of young plants near the soil surface causing it to rot. The leaves of the plant affected often turn yellow and as the plant wilts (TRFK, 2002).

2.6 Collar and Branch Canker

2.6.1 Description

Collar and branch canker is caused *P. theae* which gains access to the living and growing layer of the stems just underneath the bark but in every case there must have been

previous injury by insects, pruning, lightning or by other agents (Holiday,1980). Once it has found admission it spreads up and down the stem in the living layer underneath the bark and every now and then causes swellings that rapture the bark and come to the surface in order to fruit and produce the spores (seed) by which it is spread. The spores mature in abundance and are blown about in the atmosphere all over the estate until they fall on another injured or cut surface where it spreads and produces more fruit and spores (TRFK, 2002).

2.6.2 Symptoms of Branch and Collar Canker

Canker lesions develop on the stem at the collar region (Plate 2.1) or on the branches. Upper edges of the lesions are usually heavily callused. Leaves on branches girdled by the lesions turn yellow and ultimately the branches ultimately die (Ram, 1981). Where the lesions girdle the main stem the whole plant usually dies. The diseased areas may be regular or irregular in shape, often sunken and grey to black in colour. In instances where the branch or collar is completely girdled, a thick ridge of callus forms at the upper margin of the canker (Holiday, 1980).

Plate 2.1: Photograph of Tea Plant Infected by Branch and Collar Canker

2.6.3 Predisposing Factors to Branch and Collar Canker

The susceptibility of tea to infection by *P. theae* is thought to be influenced by the moisture availability to plants and water holding capacity of the soil (TRFK, 2001). It occurs when the bark moisture of the stem falls below a critical level. Other factors include deep planting, planting in gravely soils, mulching closer to collar, wounds caused by weeding implements, fertilizer application closer to collar, pegging, low moisture status in bark and surface watering during dry season (Anon, 1991).

2.6.4 Management of Branch and Collar Canker

Badly infected branches are pruned off below the canker lesions and the pruned cuts treated with suitable protectants for example copper oxychloride. Cultural control involves avoiding the use of susceptible clones like TRFK 301/5, TRFK 301/4, TRFK 303/1199 and TRFK 6/8, particularly in areas with history of the disease. Plants should be treated lightly during propagation, injuries to plants as a result of weeding, pegging, pruning and insects should be avoided (Mutai and Cheramgoi, 2009). Only healthy, vigorous plants with a well developed root system should be planted. Planting in poor soils, in areas where rainfall is marginal or inadequate should also be avoided (TRFK, 2002). To avoid severe moisture deficiency stress, mulching should be practiced to conserve soil moisture during dry season where possible irrigation should be practiced.

2.6.5 Distribution of Branch and Collar Canker in Kenya

The disease is apparently on the increase in some tea growing areas in Kenya. Disease surveillance in 2005 showed that there was increase in the number of stem canker reported in some farms in the East of Rift as compared to the same period in 2004 (TRFK, 2007). The incidences were reported in Kiru, Chinga, Iria-ini, Ikumbi, Kimunge and Gatungulu KTDA factory. In the West of Rift, incidences of the disease were reported in Kericho, Kisii and Nandi Districts (TRFK, 2009).

2.7 *Phomopsis theae*

*Phomopsis theae b*elongs to the Fungi imperfecti group and is closely allied to *Leptothyrium theae*. It differs from the latter in that it produces two kinds of spores or conidia namely 'A' spores and 'B' spores. The 'A' spores are spindle-shaped and closely resemble the spores of *L. theae*. The 'B' Spores are needle-shaped and slightly curved (Holiday, 1980). Fruitifications develop abundantly on the cankered bark and break through a small raised pustule somewhat pimple-like and is about the size of a pin-head. From these pycnidia sticky pcycnidiospores ooze out during moist weather in yellowish or amber-coloured long, coiled spore-borne or tendrils. Infection can occur on stems of any size by means of spore, which being sticky are most probably dispersed by rain. Since dry spores cannot penetrate undamaged bark, entry is most probably through wounds (Waller and Holderness, 1997).

2.8 Neem products

2.8.1 Neem tree

The neem tree, *Azadirachta indica*, is a tropical evergreen tree native to the Indian sub continent. It occurs in medium to large size and has dark to brown grey bark and a dense rounded pinnate leaves. Neem is a source of ecofriendly insecticides, pesticides and agrochemicals (Brahmachari, 2004). Neem tree (*Azadirachta indica*), a large tree of India, has been used for centuries as insecticides, fungicides, anticonceptionals in alternate medicine (Chaturvedi *et al*., 2003). Some extracts from neem plant have been shown to be toxic to fungal pathogens such as *Poria monticolad* infecting wood (Dhyani *et al*., 2004), *Apergillus flavus* from soya bean seeds (Krishriamurthy *et al.*, 2008),

Pyricularia oryzae infecting rice plant in the field and the harvested rice (Amadioha, 2000). Antifungal, antibacterial and insecticidal components, Azadirachtin, limonoid and terpernoids have been extracted from seeds and leaves of neem (Dai *et al.*, 2001, Jarvis and Morgan, 2000). Azadirachtin has demonstrated antifungal activity (Natarajan *et al*., 2002) and two other neem components, nimbin and nimbidin, have been found to have antifungal activity. Commercially available neem formulations like Achook, Bioneem, Nimbecidine and Neemark, have shown antifungal activity against pathogenic fungi namely; *Fusarium oxysporium, Altanaria solani*, *Curvulata lunata*, *Helminthosporium sp and Sclerotium rolfsii* (Bhonde *et al.*, 1999).

2.8.2 Nimbecidine

This is a neem-oil-based pesticide which contains Azadirachtin as an active ingredient and many other active compounds like meliantrol, slanin, and nimbin. Azadirachtin is a complex tetra norto-terpenoid limonoid from the neem plant, is the main component responsible for antifeedant, growth inhibitory, growth regulatory, and toxic effects on insects (Aerts and Mordas, 1997; Koul *et al*., 2004). It is used to control thrips, white flies in French beans, aphids and snow peas. It is also applied to control cut worms, weevils and wire worms of tomatoes (Schmutterer, 1990).

2.8.3 Trilogy 70EC

Trilogy (also packaged as Inact, Green Light Neem Concentrate and Green Light Rose Defense) is neem oil that has had the azadirachtin and at least some other components separated from it. It is also called ‘Clarified Hydrophobic Extract of Neem Oil’. It is a

multipurpose insecticide, acaricide and fungicide that control both downy and powdery mildews on cucurbits (Meister, 1999). Trilogy significantly retarded several growth parameters of the pathogen, *Podosphaera xanthii*, namely multiple germ tube formation, number of germ tubes haustoria and colony size on cucumber leaves (Aboellil, 2007).

2.9 *Warburgia ugandensis*

Warburgia ugandensis is a highly aromatic evergreen tree (Plate 2.2) within the family Canallacae with a characteristic bitter and peppery taste (Wube *et al.*, 2005). It is 15-30 m tall, 70 cm in diameter, with a bark that is smooth or scaly and pale green or brown. It is endemic to East Africa and widely distributed in lower rain forest and drier highland forest areas at altitudes between 1000 m and 2000 m (Maundu and Tengnas, 2005). This tree species has a high pharmaceutical value, both for humans and livestock, exhibiting a broad spectrum antimicrobial activity (Olila *et al*., 2001). Warburganal and muzigadical have been characterized as the active compounds against fungi. Several chemical and pharmacological studies conducted to investigate extracts from *Warburgia* species have confirmed presence of antifungal, antiulcer, insect antifeedant, molluscal, antimycobacterials and antileishamanial active sesquiterpenes (Wube *et al.*, 2005; Ngure *et al.*, 2009). The antifungal and antibacterial activity of warburganal, polygodial and muzigadial against a range of organisms, including *Staphylococcus aureus, Pseudomonas aeruginosa, Candida utilis, Bacillus subtilis* and *Escherichia coli*, has been demonstrated. The bark is highly over harvested for antifungal and antibacterial properties (Akwartulina *et al*., 2011).

Plate 2.2: Photograph of *Warburgia ugandensis* tree

CHAPTER THREE

MATERIALS AND METHODS

3.1.1 Study Site

The study was carried out in Ainamoi village, Kericho County (Figure 3.1). The research was done in the laboratory of Tea Research Foundation of Kenya (TRFK) in Kericho County. Kericho County lies in the Lake Victoria basin. Its geology is characterized by volcanic as well as metamorphic complexes. The county receives relief rainfall with moderate temperatures of 17 ^{o} C and low evaporation rates. The temperature ranges between 29 ^{o} C and 10 ^{o} C and the rainfall pattern is such that the central part of the county where tea is grown receives the highest rainfall of about 2125 mm (TRFK, 2002). The soils are slightly acidic with a pH of 5.5 which favours growth of tea and coffee. The soil are loam, well drained and have most of the plant nutrients required for plant growth.

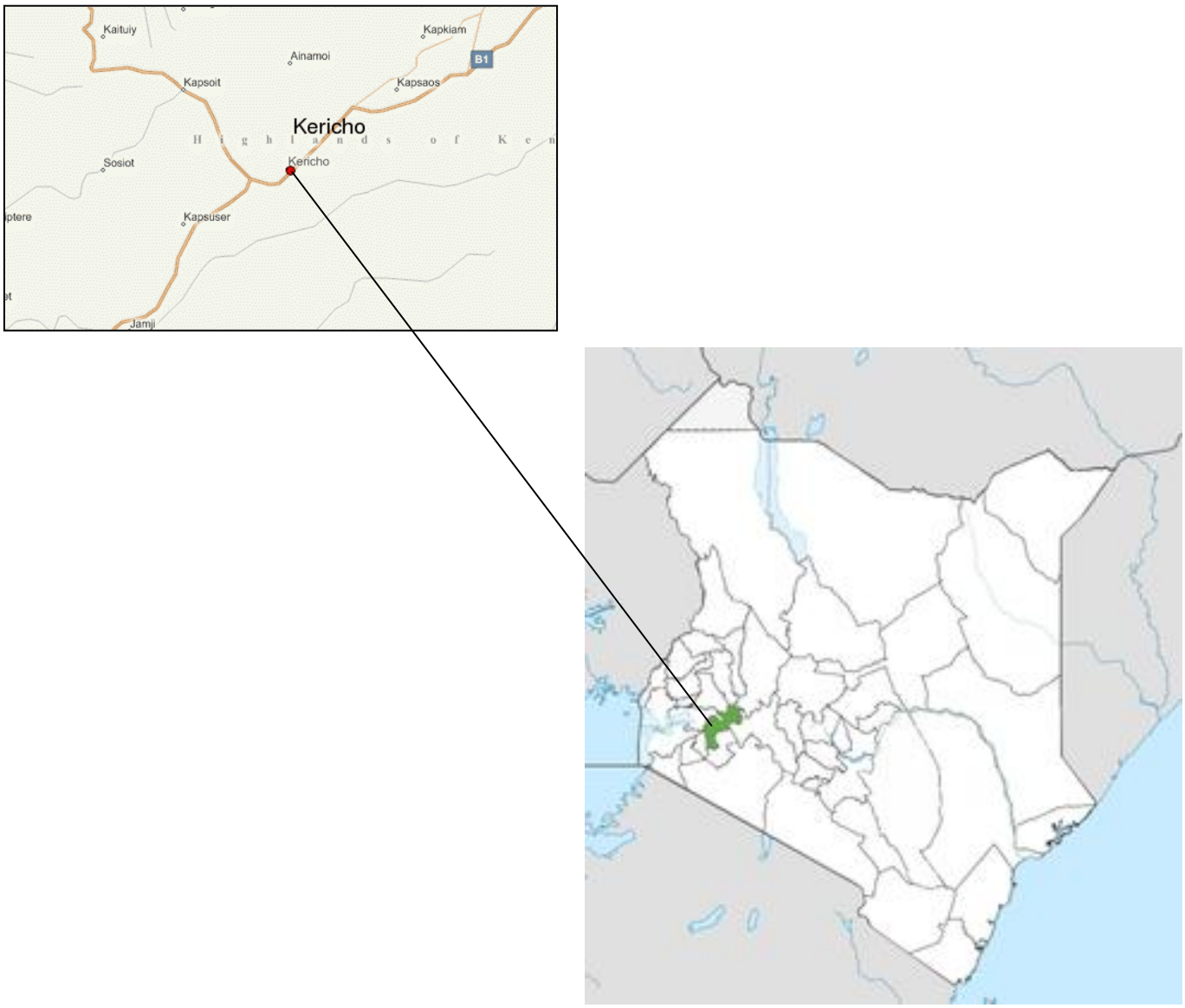


Figure 3.1: Map of Kenya Showing Location of Kericho County

3.1.2 Neem Products and Standard Fungicides Source

The neem products, Nimbecidine (Azidarachtin), manufactured by T. Stanes and Company Limited in India and Trilogy (hydrophobic neem extract) is manufactured by Certis Company in USA. They were bought from Paksons Agrochemical shop in Kericho. Standard fungicides (Saaf and Topsin) were obtained from the pesticides store in TRFK in Kericho.

3.1.3 Culture Source and Inoculum Preparation

Cultures of the pathogen, *Phomopsis theae* were obtained from the stock kept at TRFK in Kericho. Plugs of mycelium of *P. theae* were aseptically obtained from PDA agar slants of stock cultures using a flame sterilized needle and transferred to the centre of PDA medium in 9 cm diameter Petri dishes. One piece was placed in each of the dishes and incubated at 18 – 22 ^{o} C for 7 days. The inoculations were carried out in a sterile lamina flow hood for aseptic conditions.

3.2 Efficacy of Neem Products on Solid Media

Three point nine grams (3.90 g) of PDA were dissolved in each of the seventeen 250 ml conical flasks containing 100 ml of distilled water and sterilized by autoclaving at 121 ^{o} C for 20 minutes at 15 psi. The fungicides were then incorporated into the medium at the rate of 0.05 ml, 0.13 ml, 0.25 ml and 0.50 ml for Nimbecidine and 0.53 ml, 1.33 ml, 2.67 ml and 5.33 ml for Trilogy to make 10, 25, 50, and 100 ppm respectively.

3.2.1 Inoculations Using Agar Dilution Method

The amended media in each Petri dish was centrally inoculated with 2 mm mycelia discs cut from margins of three day old cultures of *P. theae* growing on PDA medium, using flame sterilized cork borer. The Petri dishes which contained unamended PDA acted as controls. The inoculated Petri dishes were arranged in a completely randomized design with four replications on a laboratory bench. Growth of the pathogen (*P. theae*) on the amended and unamended media (controls) was assessed by measuring mycelial diameter of the pathogen at three day interval for a period of 30 days.

3.3 Efficacy of Neem Products on *P. theae* in Liquid Media

Eighteen grams (18 g) of malt extract were weighed separately using analytical balance and dissolved in 1000 ml conical flasks containing 600 ml of sterilized water with 0.3 g of peptone. The neem products were incorporated into the media at 3.36 ml, 6.72 ml, 13.40 ml, and 26.80 ml for Nimbecidine and at 0.08 ml, 2.10 ml, 4.30 ml and 8.95 ml for Trilogy and were also sterilized at 121 ^{o} C for twenty minutes at 15 psi using an autoclave. The conical flasks without the products and fungicides were set up. They were agitated thoroughly using a magnetic stirrer hot plate 400 (Gallen kamp) at 500 revolutions per minute. One hundred and fifty millimeters (150 ml) of each medium were measured using a sterile measuring cylinder into 250 ml conical flasks.

3.3.1 Inoculation in Malt Extract Liquid Media

A method described by Onsando (1988) to study morphology of *Armillaria mellea* was used. Mycelia from culture of *A. mellea* on MEA was used to inoculate petri dishes of

agar. A sterile loop was used to excise 3-5 mm mycelia that were placed into the centre of a petri dish. Cultures were incubated at 25 ^{o} C for 3-6 weeks. Inoculation with the P228 isolate was carried out in a lamina flow hood. Using a sterile 2 mm diameter cork borer, mycelial agar discs were cut from the margins of colonies of three day culture of *P. theae* isolate. Four discs were placed in each conical flask using a sterile inoculating needle. The needle was sterilized by dipping it in 70 % ethanol and flaming on a Bunsen burner. The conical flasks were incubated at room temperature on an orbital shaker (Gallen kamp SGM 300 SLUO) at 40 revolutions per minute for three weeks for the purpose of aeration. To obtain dry fungal biomass, the fungal biomass was harvested using Whatman's filter paper previously dried to constant weight. The harvested mycelium along with the filter paper were dried to constant weight and the weight of each fungal biomass determined.

3.4 *In vitro* Screening of Stem Bark, Root and Leaf Extracts of *Warburgia ugandensis* for their Antifungal Properties Against *P. theae*

3.4.1 Screening Using Agar Diffusion Method

The *W. ugandensis* samples were chopped into small pieces. Ten, fifteen and twenty grams of leaves, bark and roots were weighed separately and put into 250 ml Pyrex conical flasks which contained 150 ml de-ionized water. The samples were infused by autoclaving at 121 ^{o} C for 20 minutes at 15 psi using an autoclave (Gallen Kamp). Thereafter, filtration was done and 150 ml of the filtrate transferred into 250 ml conical flask containing 5.85 g plain agar. These were then sterilized by autoclaving at a temperature of 121 ^{o} C for 20 minutes. Measurements of 20 ml of warm autoclaved agar-infusion mixture were poured into each of 9 cm diameter sterile plastic disposable Petri

plates (BS 611) in a lamina flow hood. Two millimeter diameter mycelia agar disks were cut from the margins of 7-10 days old colonies of the *P. theae* isolates cultures using a 2 mm diameter cork borer. The discs were centrally placed using a transfer needle into the 9 cm diameter plastic Petri dishes containing the leaf-agar, root-agar and stem bark-agar infusion and sealed with parafilm. Three leaf extracts, three stem bark extracts and three root extracts constitute nine treatments and plain agar served as a control. All treatments were replicated four times in a completely randomized design and incubated at room temperature. The radial colony diameter was measured at an interval of 48 hours for a period of three weeks.

3.4.2 Screening in Liquid Media

A method described by Onsando (1987) was used. Sixty grams of fresh leaves stem barks and roots of *Warburgia ugandensi*s were transferred into 1000 ml sterilized labeled Pyrex conical flasks containing 600 ml of de-ionized water. The leaves, stem bark and roots were infused at 121 ^{o}C for 20 minutes at 15 psi. Using a sterile measuring cylinder, 150 ml infusion of the leaves, bark and roots were filtered aseptically with a sterilized glass funnel plugged with a small cotton wool into four 250 ml Pyrex conical flasks to make four replicates. A different funnel was used for each to avoid contamination. Inoculation, incubation and harvesting were carried out as described in the previous experiment, 3.3.1.

3.4.3 Harvesting of Mycelium

Fungal mats were harvested after 3 weeks by filtering using Whatman`s filter paper previously dried to constant weight and weighed together with agar blocks that formed

part of the inoculums. The harvested mycelia along with the filter paper were dried in an oven (Menimen) at 60 ^{o} C to constant weight and weight of each fungal biomass plus the filter paper measured using an analytical balance. The dry weight of each biomass was determined.

3. 5.1 Effect of Neem Product, Standard Fungicides and *W. ugandensis* Extracts on Growth of *P. theae* Using Agar Diffusion Method

One point five grams (1.5 g) of PDA were dissolved in 100 ml of de-ionized water in 250 ml Pyrex conical flasks and sterilized by autoclaving at 121 ^{o} C for 20 minutes at 15 psi. The most active neem product (Nimbecidine) was incorporated into the medium at different rates to make 10, 25, 50, and 100 ppm. The fungicides were also incorporated into the medium at 0.05 ml, 0.13 ml, 0.25 ml and 0.50 ml for Topsin and 0.53 ml, 1.33 ml, 2.60 ml and 5.33 ml for Saaf to make 10, 25, 50 and 100 ppm respectively. The most effective of the *W. ugandensis* (bark) samples were chopped into small pieces, and then 10, 15 and 20 g weighed and put into 250 ml Pyrex conical flask containing 150 ml of de-ionized water. They were then infused at 121 ^{o} C for 20 minutes at 15 psi. The infusion was filtered and 100 ml transferred into 250 ml conical flask containing 1.5 g of plain agar and autoclaved at 121 ^{o} C for 20 minutes. The agar-infusion, neem product-medium and fungicides-medium mixtures were agitated thoroughly using a magnetic stirrer hot plate, at 500 revolutions per minute. Approximately 20 ml of the mixture were poured into each of 9 cm diameter sterile disposable Petri dishes in a lamina flow hood. Inoculation, incubation and harvesting were done as in the previous experiment, 3.2.1.

3.5.2 Effect of Neem Product, Standard Fungicide and *W. ugandensis* Extract on Growth of *P. theae* Using Broth Dilution Method

Eighteen grams of malt were weighed separately using analytical balance then dissolved in sixteen conical flasks containing 600 ml of sterilized water with 0.3 g of the peptone. The most active neem product (Nimbecidine) and fungicides (Saaf and Topsin) were incorporated as in section 3.3, while the most effective *W. ugandensis* extract (bark) was incorporated as in section 3.4.2. Inoculation, incubation and harvesting were done as in the previous sections 3.3.1 and 3.4.3.

3.6 Data Analysis

All the results were subjected to Analysis of Variance (ANOVA) using GMSTAT software to test for significant difference in radial growth and mycelial weights of *P. theae,* among the standard fungicides (Topsin and Saaf), neem products (Nimbecidine and Trilogy), *Warburgia ugandensis* extracts (leaves, bark and roots) and control. Separation of means was carried out using Tukeys` test at $P \leq 0.05$.

CHAPTER FOUR

RESULTS

4.1 *In vitro* Efficacy of Neem Products (Trilogy and Nimbecidine) on Growth of *Phomopsis theae* in Solid Media

For Nimbecidine, a neem product, the radial measurements in 10 ppm, were 2.00 mm, 3.50 mm, 7.00 mm and 12.50 mm for day 6, 15, 21 and 30 respectively. At the concentration of 25 ppm, the measurements were 2.00 mm, 2.00 mm, 7.25 mm and 10. 00 mm for day 6, 15, 21 and 30 respectively. The measurements in day 6, 15, 21 and 30 were 2.00 mm, 2.00mm, 3.00 mm and 4.75 mm. while in 100 ppm, they were 2.00 mm for day 6, 15 and 21 and 3.56 mm in day 30 (Table 4.1). In Trilogy, the radial measurements were 2.00 mm, 8.73 mm, 39.50 mm and 62.00 mm in the concentration of 10 ppm, for day 6, 15, 21 and 30 respectively. In 25 ppm, the measurements 2.00 mm, 7.25 mm, 38.25 mm and 56.25 mm in the days of the experiment. In the concentration of 50 ppm, the measurements were 2.00 mm, 4.00 mm, 10.25 mm and 35.00 mm in 100 ppm (Table 4.1).

At the lower concentration, 10 ppm, all the treatments were significantly similar ($P \leq 0.05$, df=79) on day 6 and 15. However, on day 21, Trilogy was seen to differ significantly from Nimbecidine (Table 4.1). At 25 ppm, no significant difference was seen on day 6 in various treatments. On day 15, there was no significant difference between Trilogy and control ($P \leq 0.05$, df= 79), while there was significantly different between Trilogy and Nimbecidine. On day 21, the measurements for radial growth of *P. theae* in media amended with Nimbecidine and Trilogy were 7.25 mm and 38.25 mm respectively. Trilogy was seen to be significantly different from Nimbecidine treatment.

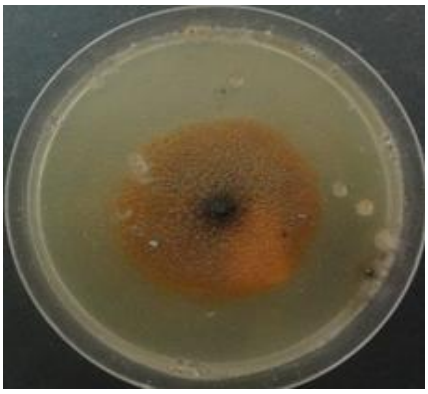
In the concentration of 50 ppm, all the treatments did not differ from each other. Similarly, on day 15, there no significant differences ($P \leq 0.05$) among the treatments. However on day 21 and 30 Trilogy differed from Nimbecidine treatment (Table 4.1). In 100 ppm, no significant differences were noted on days 6 and 15 in all the treatments ($P \leq 0.05$). On days 21 and 30 radial measurements of *P. theae* in media amended with Trilogy were 9.00 mm and 27.00 mm which were significantly superior ($P \leq 0.05$, df=79) to that in media amended with Nimbecidine which were 2.00 mm and 3.56 mm respectively. In the media amended with Nimbecidine and Trilogy there was higher percentage of inhibition compared to that of the unamended media (control) (Plate 4.1, Table 4.1).

Inhibition of fungal growth by Nimbedicine was increased with the increase in concentration hence rates of inhibition of 95.33, 93.77, 86.6 and 83.67, at 100, 50, 25 and 10 ppm respectively were recorded. Comparatively, Trilogy also inhibited growth though in small percentages (19, 56.26, 53.60 and 49.50) unlike in Nimbecidine. Percentage inhibition also increased with increase in the concentrations of 10 ppm, 25 ppm, 50 ppm and 100 ppm (Table 4.1).

Table 4.1: Radial Measurements and Percentage Inhibition of *P. theae* Growing on Media Amended with Neem Products (Trilogy and Nimbecidine) on Days 6, 15, 21 and 30 after Treatment

Treatments	Mean radial measurements of *P. theae* (mm) Number of Days				% inhibition
	6	15	21	30	
Trilogy 10 ppm	2.00a[1]	8.73ab	39.50a	62.00b	19.00
25 ppm	2.00a	7.25ab	38.25a	56.25c	56.26
50 ppm	2.00a	4.00ab	10.25b	35.50b	53.60
100 ppm	2.00a	4.00ab	9.00b	27.00c	49.50
Nimbe 10 ppm	2.00a	3.50ab	7.00bc	12.50f	83.67
cidine 25 ppm	2.00a	2.00b	7.25cd	10.00f	86.60
50 ppm	2.00a	2.00b	3.00c	4.75g	93.77
100 ppm	2.00a	2.00b	2.00c	3.56g	95.33
Control	2.00a	12.50a	41.50a	76.25a	0.00

[1]Mean values in the same column followed by similar letters are not significantly different at $P \leq 0.05$



Nimbecidine

Trilogy

Control

Plate 4.1: *P. theae* growing in media amended with Neem products (Nimbecidine and Trilogy) and control.

4.2 Efficacy of Neem Products on Growth of *P. theae* in Liqiud Media

Among the neem products, higher mycelial weights were recorded in Trilogy compared to Nimbecidine (Plate 4.2). Among the various concentrations of Trilogy tested, the weights recorded were 1.47 g, 1.45 g, 1.20 g and 0.87 g in 10 ppm, 25 ppm, 50 ppm and 100 ppm respectively (Table 4.2). In the media amended with Nimbecidine, mycelial weights of 0.72 g, 0.66 g, 0.61 g and 0.43 g in 10, 25, 50 and 100 ppm were recorded

though they were not significantly different ($P \leq 0.05$) from each other (Table 4.2). At the lower concentration (10 ppm), Nimbecidine significantly suppressed the growth of the pathogen compared to Trilogy, though the later was seen to inhibit growth more than in the control. In the concentration of 25 ppm, Trilogy was significantly higher ($P \leq 0.05$) than those of Nimbecidine and significantly lower than the control. In the concentration of 50 ppm of Nimbecidine and Trilogy were 0.61 g and 1.20 g respectively (Table 4.2). The mycelial weights in Nimbecidine were significantly lower ($P \leq 0.05$) than in Trilogy and also in the control. At a higher concentration (100 ppm) Nimbecidine and Trilogy were significantly different ($P \leq 0.05$) from each other. Nimbecidine inhibited growth more than Trilogy but were not significantly different from ($P \leq 0.05$) each other but from the control. In all the concentrations, Nimbecidine was seen to significantly suppress ($P \leq 0.05$) the growth of the pathogen compared to Trilogy (Table 4.2).

Table 4.2: Mycelial Weights of *Phomopsis theae* in Liquid Media Amended with Neem Products (Nimbecidine and Trilogy).

Treatment	Mycelial weights in grammes at different concentrations			
	Concentration (ppm)			
	10	25	50	100
Trilogy	1.47b[1]	1.45b	1.20bc	0.87cd
Nimbecidine	0.72de	0.66de	0.61de	0.43de
Control	2.16a	2.16a	2.16a	2.16a

[1]Mean values in the same column followed by similar letters are not significantly different at $P \leq 0.05$

Plate 4.2*:* Orbital shaker with *P. theae* growing in liquid media amended with neem products.

4.3 Efficacy of the *Warburgia ugandensis* Extracts (leaf, root and stem bark) on the Growth of *P. theae* in Solid Media

The radial growth of *P. theae* in the media amended with bark extracts was 2.00 mm in all the rates throughout the experimental period. However, on day 6 and 15, the radial measurements were not significantly different from the other extracts. On day 21 and 30, radial measurements in the bark extracts were significantly lower ($P \leq 0.05$) than that of the root and leaf extracts (Table 4.3). The media amended with root extracts, also showed inhibition of the growth of the pathogen.

On the 6th day, the radial measurements were 2. 00 mm for the three rates 10 g, 15 g, and 20 g (Table 4.3). On day 15, radial measurements were 8.70 mm, 6.80 mm and 5.00 mm and were not significantly different ($P \leq 0.05$) from each other. For the 21st day, the

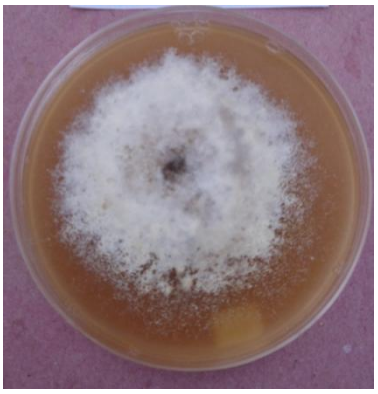
measurements were 33.30 mm, 28.80 mm, and 9.70 mm in 10 g, 15 g and 20 g respectively. There was no significant difference ($P \leq 0.05$) between the lower rates 10 g and 15 g, but were different from the higher rate (20 g). On day 30, radial measurements were 66.00 mm, 59. 00 mm and 15. 00 mm, for 10 g, 15 g and 20 g respectively.

There was significant difference ($P \leq 0.05$) among the three rates with the higher rate being significantly lower than the other rates. All the rates showed significant difference ($P \leq 0.05$) from that of the control. In the media amended with leaf extracts, the radial measurements on day 6 were 2.00 mm in each of the rates, 10 g, 15 g and 20. They were not significantly different ($P \leq 0.05$) from each other. On day 15, the radial measurements were 11.30 mm, 12. 50 mm and 13,00 mm in 10 g, 15 g and 20 g respectively, (Table 4.3). There was no significant difference ($P \leq 0.05$) among them. For day 21, radial measurements were 55.80 mm, 54.00 mm and 47.80 mm in 10 g, 15 g, and 20 g. the lower rate was significantly different ($P \leq 0.05$) from the higher rate. All the rates were significantly lower ($P \leq 0.05$) than that of the control. On day 30, in the three rates had radial diameter was 85.00 mm and were significantly higher ($P \leq 0.05$) than the control.

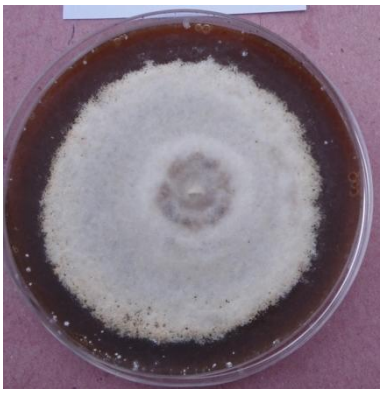
At the lower rate (10 g), there was no significant difference among the radial measurements in all the extracts, bark, root and leaf. On the 6^{th} day and 15^{th} day they were not significantly different ($P \leq 0.05$) from that in the control (Table 4.3). On the 21^{st} day, the radial measurements in the media amended with bark extracts were significantly lower ($P \leq 0.05$) than in media amended with root, leaf extracts and in the control. The measurements in the root extracts were not significantly different ($P \leq 0.05$) from that of

the control. The radial measurements in the leaf extracts were significantly superior among all the extracts. On the 30th day, the measurements in bark extracts were also significantly lower ($P \leq 0.05$) than in the media amended with the other extracts and also in the control. The measurements n the root extracts were significantly lower than in the leaf and control, which were significantly different ($P \leq 0.05$) from each other.

In the media amended with 15 g of the extracts, there was no significant difference among the measurements in the bark, root and leaf extracts on day 6 and 15. On day 21, the mycelial measurements in the bark were significantly lower ($P \leq 0.05$) than in the root extracts, leaf extracts and the control. Similarly the radial measurements were significantly lower ($P \leq 0.05$) than in the leaf extracts and the control. The measurements in the leaf extracts were significantly higher ($P \leq 0.05$) than in the control (Table 4.3). On the 30th day, the radial measurements in the media amended with leaf extracts were significantly the highest ($P \leq 0.05$) among all the treatments while the measurements in the bark were the lowest. For the highest rate (20 g) of the extracts, the radial measurements on day 6 and 12 were not significantly different ($P \leq 0.05$) from each other and that in the control. On day 21, the measurements in the root were significantly lower ($P \leq 0.05$) than those in the leaf and control and higher than the measurements in the bark extracts. On day 30, the measurements in the bark extracts were significantly the highest among the extracts and control. The measurements in the leaf extracts were significant lowest ($P \leq 0.05$) even compared to the control (Plate 4.3). The measurements in the media amended with root extracts were significantly lower than in the control.



Root Extracts



Leaf Extracts

Control

Plate 4.3: *P. theae* growing on media amended with *W. ugandensis* extracts and control

Table 4.3: Radial Measurements of *P. theae* Growing on Media amended with Leaf Root and Bark Extracts of *Warburgia ugandensis* on days 6, 15, 21 and 30

Treatment (g)		Mean radial mycelial measurements of *P. theae* (mm) Number of days				
		6	15	21	30	% inhibition
Bark	10	2.0a[1]	2.0a	2.0f	2.0f	97.27
	15	2.0a	2.0a	2.0f	2.0f	97.27
	20	2.0a	2.0a	2.0f	2.0f	97.27
Root	10	2.0a	6.8a	33.3cd	66.0c	9.96
	15	2.0a	8.7a	28.8d	59.0d	19.50
	20	2.0a	5.0a	9.7e	15.5e	78.85
Leaf	10	2.0a	11.3a	55.8a	85.0a	-15.96
	15	2.0a	12.5a	47.8b	85.0a	-15.96
	20	2.0a	13.0a	54.0a	85.0a	-15.96
Control		2.0a	12.5a	39.3c	73.3b	0

[1]Mean values in the same column followed by similar letters are not significantly different at $P \leq 0.05$

Key: g- grams per 150 ml of water

4.4 Efficacy of *W. ugandensis* Extracts (leaf, root and bark) on Growth of *P. theae* in Liquid Media

In the media amended with bark extracts, there was complete inhibition of growth of *P. theae* hence the least mycelial weights recorded. In 10 g, 15 g and 20 g of the extracts mycelial weights were 0.02 g which was significantly lower than that of the other extracts and the control. The root extracts also inhibited the pathogen to certain degree. The mycelial weights in 10 g, 15 g and 20 g were 0.95 g, 0.92 g, and 0.28 g respectively. The higher rate was significantly different from that of 10 g and 15 g and was not different from that of the bark. In the leaf extracts, mycelial weights in 10 g, 15 g and 20 g were 1.31 g, 1.19 g and 1.0 g respectively. They were not significantly different from each other.

The mycelial weights of *P. theae* in 10 g of the bark extracts was 0.02 g, while in the root and the leaf it was 0.95 g and 1.34 g respectively. The mycelial weights in the root and the leaf were not significantly different from each other. The bark differed from both the root and leaf and also from the control. At the rate of 20 g, the mycelia weights of the pathogen were 0.02 g, 0.28 g and 1.0 g in media amended with bark, root, and leaf extracts respectively. The mycelial weights in that of the bark and root did not differ significantly from each other. Leaf extracts were significantly higher than that of the root and bark extracts. Though the bark extracts had the lowest mycelial weights, the lower rate did not differ significantly from the higher rate of the root extracts. Mycelial weights of the pathogen in the media amended with 15 g and 20 g of the leaf extracts were not significantly different from that of 10 g of the root extracts. The unamended media had

mycelial weight of 2.31 g and was significantly different from that of the bark, root and leaf extracts.

Table 4.4: Mycelial Weights of *P. theae* in Liquid Media Amended with *Warburgia ugandensis* Extracts, from the Bark, Root and Leaf at Different Rates.

Treatment	Mycelial weights in grammes in different rates of extracts		
	Rates (g)		
	10	15	20
Bark	0.02d[1]	0.02d	0.02d
Root	0.95c	0.92c	0.28d
Leaf	1.34b	1.19bc	1.0bc
Control	2.31a	2.31a	2.31a

[1]Mean values in the same column followed by similar letters are not significantly different at $P \leq 0.05$

Key: g- Grams per 150 ml of water

4. 5 *In vitro* Efficacy of the Most Effective Neem Product (Nimbecidine), Standard Fungicides (Topsin and Saaf) and the Most Effective *W. ugandensis* Extracts (bark) on Growth of *P. theae*

In the media amended with standard fungicides Topsin and Saaf, the mean radial measurements of *P. theae* were 2.00 mm in all the concentrations (10 ppm,25 ppm, 50 ppm, 100 ppm) throughout the experimental days (6, 12, 24 and 30). These measurements were similar to those of the media amended with bark extracts of *W. ugandensis*. The percentage inhibition of the growth was 97.61 for Topsin, Saaf and bark extracts (Table 4.5).

Growth of *P. theae* in Nimbecidine was significantly higher than in fungicides and bark extracts on days 12, 24 and 30, but was significantly lower than in the control (Table 4.5). The growth of the fungus in the higher concentration (50 ppm and 100 ppm), was significantly lower than in the lower concentrations (10 ppm and 25 ppm) from day 12 to day 30 and significantly lower than the growth in the control (Table 4.5). Growth in the control was significantly highest throughout the experiment, while in Topsin, Saaf and bark extracts, it was significantly lowest.

Growth inhibition was highest in Topsin, Saaf and bark extracts but not different in percentage values. They all inhibited at 97.61 percent (Table 4.5). In Nimbecidine, growth inhibition varied with the concentration, with the highest concentration inhibiting the growth at 94. 33 percent, followed by 89.25 percent at 50 ppm, 85.67 percent at 25 ppm and 81.50 percent at 10 ppm. However, there was 0 percent inhibition in the control (Table 4.5).

Table 4.5: Radial Measurements of *P. theae* Growing on Media Amended with Standard Fungicides (Topsin and Saaf), Neem Product, Nimbecidine, and *W. ugandensis* Extract, (bark) on days 6, 12, 24 and 30

Treatment		Means radial measurements of *P. theae* (mm)				% inhibition
		Number of Days				
		6	12	24	30	
Topsin	10 ppm	2b[1]	2c	2d	2d	97.61
	25 ppm	2b	2c	2d	2d	97.61
	50 ppm	2b	2c	2d	2d	97.61
	100 ppm	2b	2c	2d	2d	97.61
Saaf	10 ppm	2b	2c	2d	2d	97.61
	25 ppm	2b	2c	2d	2d	97.61
	50 ppm	2b	2c	2d	2d	97.61
	100 ppm	2b	2c	2d	2d	97.61
Nimbe	10 ppm	2b	7.25b	12.75b	15.5b	81.50
cidine	25 ppm	2b	6b	10b	12b	85.67
	50 ppm	2b	3c	7.25c	9c	89.25
	100 ppm	2b	2c	4.25cd	4.75cd	94.33
Bark	10 g	2b	2c	2d	2d	97.61
	15 g	2b	2c	2d	2d	97.61
	20 g	2b	2c	2d	2d	97.61
Control		12.5a	29.75a	77a	83.75a	0

[1]Mean values in the same column followed by similar letters are not significantly different at P≤0.05

4.6 Efficacy of the Most Effective Neem Product (Nimbecidine), Standard Fungicides (Topsin and Saaf) and the Most Effective *Warburgia ugandensis* Extracts (bark) in Liquid Media

Topsin, Saaf and bark extracts inhibited growth in all the concentrations hence mycelial weights of 0.02 g were recorded after incubating for three weeks (Table 4.6). There was no significant difference among the weights in these media (Table 4.6). For Nimbecidine, growth was higher than in the media amended with fungicides and bark extracts (Table 4.6). There was increase in inhibition with increase in the concentration of the neem product, but there was no significant difference among them. Mycelial weights were significantly lower than those in the control in all the concentrations. The weights in the control were significantly highest compared to those in the amended plates (Table 4.6).

Table 4.6: Mycelial Weights of *Phomopsis theae* Grown in Liquid Media Amended with Neem Products (Nimbecidine), Standard Fungicides (Topsin and Saaf) and *Warburgia ugandensis* (bark) Extracts

Treatment	Mycelial weights in grammes at different concentrations after three weeks of incubation			
	Concentration (ppm)			
	10	25	50	100
Topsin	0.02c[1]	0.02c	0.02c	0.02c
Saaf	0.02c	0.02c	0.02c	0.02c
Nimbecidine	0.74b	0.66b	0.62b	0.45b
Bark	0.02c	0.02c	0.02c	0.02c
Control	2.31a	2.31a	2.31a	2.31a

[1]Mean values in the same column followed by similar letters are not significantly different at $P<0.05$

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

5.1.1 Efficacy of Neem Products, (Nimbecidine, Trilogy) on Growth on *P. theae*

The study revealed that Nimbecidine significantly inhibited growth of *P. theae* more than the other neem product both in solid and liquid media (Table 4.1 and 4.2). Nimbecidine contains compounds which have antifungal activity. These compounds might include azadirachtin, meliantrol, slanin and nimbin. Dubey and Kumar (2009) reported that the fungicidal effect of azadirachtin was as good as the fungicides bavistin and mancozeb. It has been shown to have significantly inhibited the growth of plant pathogenic fungi such as *Fusarium oxysporum, Rhizoctonia solani Altenaria solani* and *Sclerotina sclerotiorum* (Moslem, 2009). Similar results were reported by Moline and Locke (1993) who found out that neem oil had some fungicidal activity towards some post harvest apple decay fungi such as *Botryis cinera* ex. Fr, (gray mold) and *Glomerella cingulata (*anthracnose fungus). Furthermore, Bohra *et al.* (2006) reported that neem has active components such as azadirachtin, nimbin and nimbicin which are antifungal in nature. Several studies have shown that neem oil produced negative effects on *Beauveria bassiana*, by inhibiting germination, colony diameter and conidiogenesis were seen (Hirose *et a*l., 2001). Other results by Babu *et al*. (2000), recorded that spraying with 3 % neem oil in tomato pot cultures resulted in 53 % reduction in disease the incidence compared to the control while Patil *et al.* (2000), found out that incidence of tomato early blight caused by *Alternaria solani* was reduced by neem oil with increased fruit yield.

Trilogy, a miticide and agricultural fungicide, showed lower inhibition of growth compared to Nimbecidine in this study (Table 4.1 and 4.2). At the higher concentration (100 ppm), it had an inhibition of 64.59 per cent in solid medium which is lower compared to the other neem product (Table 4.1). Similar results were also seen in the liquid media. The results disagree with that of Poiloakidou (2005) who reported that Trilogy was effective against *Pseudomonas xanthii* at lower concentrations in a laboratory assay. This could be because *P. xanthii* is a bacterium and therefore the observed difference could be due differences between the mode of action of the plant products on fungi and bacteria. Result agrees with that reported by Wszelaki *et al*. (2002) who found out that Trilogy had no effect on early blight and septoria leaf spot disease control that are caused by *Alternaria solani* and *Septoria lycopersi* respectively. Data that is available regarding the use of Trilogy indicates that Trilogy manages diseases in curcurbits to a limited extent (Meister, 1999).

In other crops Trilogy provides no disease control, an observation sister to the position depicted by the data recorded from the study (Table 4.1 and 4.2). Neem oil failed to suppress spot anthracnose on leaves of dogwood (Hagan and Akridge, 2007). Seaman *et al*. (2006), reported that Trilogy failed to suppress foliar diseases caused by *A. solani* and *Septoria lycopersici.* Another investigator, Aboellil (2007) reported that a natural product from *A. indica*, Trilogy, significantly inhibited many growth parameters of cucumber powdery mildew pathogen (*Podosphaera xanthii*) and induced resistance in cucumber plants. The difference in the activities of the two neem products could be due to their

active ingredients. Nimbecidine has 0.03 % azadirachtin while Trilogy which is hydrophobic extract having less than 0.03 % azadirachtin.

5.1.2 Efficacy of *Warburgia ugandensis* Extracts on Growth of *P. theae*

The fungicide properties of the bark agreed with the investigation previously done. Several chemical and pharmacological studies conducted to study extracts from the bark of *Warburgia* species have confirmed presence of antifungal, antiulcer, insect antifeedant, molluscal, antimycobacterial and antiheshamanial active sesquiterpenes, (Lunde and Kubo 2000; Wube *et al*., 2005; Ngure *et al*., 2009). Epipolygodial, mannilol, muzigadial, polygodial, tannin and warbuganal are examples of alkaloid group of metabolites present in the bark of *W. ugandensis* (Bekalo *et al*., 1996). A range of biological effects such as trypanocidal, antiviral, fungicidal and antibacterial activity has been ascribed to them.

W. ugandensis extracts have been previously tested and found to exhibit a broad sprectrum antimicrobial activity against a range of microorganisms including *Candida utilis*. They are also said to have antimicrobial activity against *F. oxysporum, F. solani, Alternaria* spp, *R. stolonifer, A. niger, R. solanacearum* and *S. ipomoeae,* which are soil pathogens associated with rotting of sweet potato and other root crops (Ristaino, 1993). This suggests that the pathogens can be managed using herbal extracts as had also been observed in other studies (Okigbo and Nmeka, 2005).

Leaf extracts were least effective in inhibiting the growth of the pathogen. In the three rates, radial growth was not significantly different from each other. The results disagreed with those of Oniango (2003) who reported that leaf extracts of *W. ugandensis* gave maximum inhibition of growth of *P. theae*, even in the lowest concentration. The difference between the observation from the current study and that of Oniango (2003) could be due to the solvents used for extraction which may not have captured all the active ingredients from the plant extracts. Olila *et al*. (2001), has demonstrated that this plant has both antibacterial and antifungal activities. The results show that the antifungal effects in *W. ugandensis* extracts are dependent on the core and part of the plant. This is agreement with work done by Olila (1993). This could be due to the difference in concentrations of the various active metabolites in the different parts of the plant.

5.1.3 Efficacy of the Most Effective Neem Product (Nimbecidine), Standard Fungicides (Topsin and Saaf) and the Most Effective *Warburgia ugandensis* Extracts (bark) on the growth of *P. theae*.

The results of the study that compared the efficacy of neem product Nimbecidine, bark extracts and fungicides (Table 4.5 and 4.6) indicated that bark extracts inhibited growth of *P. theae* at all the rates studied. They inhibited growth even at a lower concentration. The antifungal effects of *W. ugandensis* have been previously demonstrated. A study in Kenya showed the extracts from the plant act against soil pathogens namely; *Fusarium oxysporum, Alternaria passiflorae* and *Aspergillus niger* (Ruggutt *et al.,* 2006).

In the neem product, Nimbecidine, a progressive increase in inhibition of the growth of the pathogen was observed with increase in the concentration (Table 4.5). Similar

observations were made with respect to effect on weight of mycelium (Table 4.6). Antifungal activity of this product has been proven in earlier studies. The antifungal activity exhibited might be due to presence of organic acids (propanic, butyric, malic, succinic and tartaric) (Hirose *et. al*., 2001). Bajan *et al*. (1998) also observed a reduction in the vegetative growth of *Beauvaria bassiana* colonies caused by the commercial neem product. Other results indicate that neem oil protected the seeds of chick pea against the fungal diseases caused by *Rhizoctonia solani, Sclerotium rolfsii* and *Sclerotim* (NRC, 1992).

Saaf is also antifungal and was seen to inhibit the growth of the pathogen in all the concentrations and throughout the 30 days of the experiment (Table 4.5). Its antifungal effects are attributed to mancozeb and carbendazim which are the active ingredients. Results from previous work done Baby and Mouli (2000), shows that among the fungicides screened, carbendazim was found to be the best in controlling thorny blight disease of tea.

The standard fungicide, Topsin, inhibited growth at all the concentrations (Table 4.5). This confirms what other researchers reported. Pathan *et al*. (2005) tested six fungicides against *Botryodiplodia theobromae*, the causal agent for mango gummosis. Topsin and Ridoml Gold were found to be most effective in controlling the disease under laboratory and field conditions.

Antifungal activity of bark extracts of *W. ugandensis* was comparable to that of control fungicides, Saaf and Topsin. They all inhibited growth of *P. theae* in all the concentrations and also both in solid and liquid media (Table 4.5 and 4.6). The neem product, Nimbecidine, was not as effective as the standard fungicides and bark extracts, but it also inhibited to some percentage.

5.2 Conclusions

From the study, the neem product Nimbecidine was found to be more effective than Trilogy in inhibiting growth of *P. theae*. Bark extracts were the most effective among the *W. ugandensis* extracts. The antifungal activity of the bark extracts was measurable to that of the studied fungicides (Saaf and Topsin). Root extracts were also active but was concentration dependent. They were most inhibiting at high concentration. The results from this study indicated that the plant products tested that is, the neem products (particularly Nimbecdine) and bark extracts from *W. ugandensis* significantly inhibited growth of *P. theae*. Therefore they have the potential to replace or supplement fungicides for use to control *P. theae* and consequently the branch and collar canker of tea caused by the pathogen.

5.3 Recommendations

i) In the case of the neem products, Nimbecidine was found to be effective in inhibiting the growth of *P. theae* as well as the bark extracts of *W. ugandensis*. The farmers can use these instead of the chemical fungicides to manage the disease.

ii) The bark extracts of *W. ugandensis* inhibited the growth of *P. theae* more than the root and leaf extracts. Therefore there is need to establish if the antifungal compounds are concentrated in the bark of *W. ugandensis* compared to the other parts or different antifungal compounds are in the different parts or different antifungal compounds are in the different parts.

iii) The antifungal activity of the bark extracts was observed to be as effective as that of the fungicides. *In vivo* studies to compare the antifungal activity of the fungicides (Saaf and Topsin) and *W. ugandensis* bark extracts is recommended.

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APPENDIX

Recipes

Malt Extract Agar (MEA)

Malt extract	20.0 g
Peptone	1.0 g
Dextrose	20.0 g
Agar	15.0 g
Distilled water	1.0 litre

Autoclaved at 121 ^{o} C

Potato Dextrose Agar (PDA)

Potato starch	4.0 g
Glucose	20.0 g
Agar	15.0 g
Distilled water	1.0 litre

Autoclaved at 121 ^{o} C